THE DEVELOPMENT OF ROSMARINIC ACID DERIVATIVES TO TARGET IL17A IN GLIOBLASTOMA MULTIFORME VIA ANGIOGENIC PATHWAYS

by

MD SHAMSUDDIN SULTAN KHAN

Thesis submitted in fulfillment of the requirements

for the degree of

Doctor of Philosophy

September 2017

DEDICATION

To My Parents, My wife, Brother, and Uncle

ACKNOWLEDGEMENT

First and for foremost, I would like to thank Allah the almighty creater for all that He has granted to be that has led me to this path of knowledge. My deepest gratitude to my parents for raising me and educating me and to my wife for being my pillar of hope and support.

My sincere appreciation to Associate Prof. Dr. Amin Malik Shah Abdul Majid for giving me the opportunity to be part of Eman Laboratory and supervising and guiding me throughout my research work. Deep appreciation is to Mrs. Rafath E Rouf, Dr. Mohamed Adnan Iqbal, Dr. Aman Shah, Prof. Roberta Fruttero and Prof. Barbara Rolando, Mr. Razzak Hamdan, Dr. Zurina Hassan, Dr. Zulkifli Mustafa, Dr. Abdus Salam, Dr. Mohamed Asif, Dr. Mohamed Khadeer Ahamed Basheer, and Dr. Rosenani Anwarul haque for their extensive contribution to my study. Together, I extend my gratitude to students and academic faculty members who have shared their thoughts with me in the experiments. I have achieved success in responding to their thoughtful suggestions. I also, acknowledge with appreciation to my colleagues who have generously provided scientific and technical information and consumables, updated information and comments for my study. I gratefully acknowledge my lab mates and academic editors who contributed to the development of my research through their critiques, reviews and suggestions. Additionally, I am thankful to EMAN Biodiscoveries Sdn Bhd, Natureceuticals Sdn Bhd for funding this project and providing access to this facilities. I am also grateful to Universiti Sains Malaysia for awarding me the USM doctoral fellowship. My deep appreciation is to my parents Mr. Md Rowshan Ali Khan and Mrs. Sjaeda Khan for their enormous support.

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$$\text{Absorbance total} = \frac{(\varepsilon_{HA-}\ \varepsilon_{A^-})\times[10^{(pH-pKa)}]}{1+\ 10^{(pH-pKa)}} \times S_t$$

$$LogD = log\left(\frac{C_{oct}}{C_{aq}}\right)$$

$$I\% = (1 - \frac{A_{sample}}{A_{blank}}) \times 100$$

$$Inhibition = 1 - \frac{absorbanceoftreated}{absorbanceofuntreated} \times 100$$

$$Proliferation = \left(\frac{absorbanceoftreated}{absorbanceofuntreated} \times 100\right) - 100$$

$$P_{app} = \frac{d_Q}{d_t} \times 1(A \times C_o)$$

$$\%inhibition\ (migration) = 1 - \frac{the\ width\ at\ the\ th\ hour}{the\ width\ at\ zero\ time} \times 100$$

$$\%\ Inhibition\ (vessel) = 1 - \frac{A_o}{A} \times 100$$

$$Fold\ Change = \frac{\frac{T_{firefly}}{T_{renilla}}}{\frac{C_{firefly}}{C_{renilla}}}$$

$$Tumor\ volume, V = \frac{a^2 \times b}{2}$$

Tumor volume (mm^3) =1/2 × L × W^2

LIST OF ABBREVIATIONS

ADME	absorption distribution metabolism excretion
AMES	a test to assess the mutagenic potential of chemical compounds
A549	lung carcinoma epithelial cells
ACD ILAB	a computational tool
AgR	silver rosmarinate; silver salt
AKT	protein kinase B
Ang-1	angiopoietin-1
Ang-2	angiopoietin-2
ANOVA	analysis of variance
BAD	Bcl-2 antagonist of cell death
BAX	Bcl-2 associated X
BAD	Bcl 2 Associated Death
BALB/c	bagg albino (inbred research mouse strain)
BBB	blood-brain barrier
Bcl-w	b cell lymphoma w
bFGF	basic fibroblast growth factor
BID	BH-interacting domain death
BRCA1	breast cancer gene 1
BRCA2	breast cancer gene 2
CD34	cluster difference 34
CNS	central nervous system
CTLA–8	cytotoxic T-lymphocyte–associated antigen
CD4	cluster of differentiation 4
CNS	central nervous system
COX-2	cyclooxygenase 2
CD31	cluster of differentiation 31
Caco-2	colorectal adenocarcinoma epithelial cells
CYP450	cytochrome P450
CuSO4	copper sulphate
CAM	chick chorioallantoic membrane

CREB	cAMP responsive element binding protein
cytoC	cytochrome c
DAKO	antibody against CD34 antigen
DNA	deoxyribonucleic acid
DMBA	7,12-dimethylbenz[a]anthracene
DMSO	dimethyl sulfoxide
DMEM	dulbecco's modified eagle medium
DBTRG MG	glioblastoma IV fibroblast cells
EA.hy926	human endothelial cell line
EPR	electron spin resonance
EC50	effective concentration of fifty
ERK	extracellular-regulated kinase
ERK1	extracellular-regulated kinase 1
EGFR	endothelial growth factor receptor
FRAP	ferric reducing antioxidant power
FAK	focal adhesion kinase
FT-IR	fourier transform infrared (spectroscopy)
FMT	fluorescence molecular tomography
FLVM	diamine rosmarinate/caffeate
FLVZ	imidazole rosmarinate/caffeate
GBM	glioblastoma multiforme
GS5	*Streptococcus mutans*
GSEA	gene set enrichment analysis
HUVEC	human umbilical vein endothelial cells
HCT 116	colorectal carcinoma epithelial cells
HTS	high throughput screening
HIA	human intestinal absorption
HPLC	high performance liquid chromatography
HBSS	hank's balanced salt solution
HSP27	heat shock protein 27
HTRA	the human protein serine protease
HPV	human papillomavirus
hERG	human ether-a-go-go gene

HIV	human immunodeficiency virus
H&E	haematoxylin and eosin
HGF	hepatocyte growth factor
HIF-1α	hypoxia induced growth factor 1α
IGFBP-1	insulin-like growth factor binding proteins-1
I-309	T cell mediated inflammatory cytokines
I-TAC	T-cell alpha chemoattractant
ICAM–1	intercellular adhesion molecule
IC50	fifty percent of inhibitory concentration
IL17A	Interleukin 17A
JNK	jun n-terminal kinase
KBr	potassium bromide
logPS	permeability-surface area
LogD	logarithm of a ratio
LOAEL	lowest observed adverse effect
LDL	low density lipoprotein
LD50	fifty percent of lethal dose
MAKNA	malaysian national cancer council
MSH2	mutS homologue 2
mTOR	mammalian target of rapamycin
mRNA	messenger ribonucleic acid
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2
MMP	matrix metalloproteinases
MAPK	mitogen-activated protein kinase
MCF7	breast adenocarcinoma epithelial cells
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MRTD	maximum tolerated dose
MDCK-MDR1	madin darby canine kidney (MDCK) cells with the *MDR1* gene
MAPK	mitogen-activated protein kinase

NMR	nuclear magnetic resonance
NaR	sodium rosmarinate; sodium salt
NOVA	a computational software program
NF-κβ	nuclear factor kappa beta
NOTCH	a human gene encoding a single-pass transmembrane receptor.
NIH	national institute of health (USA)
OECD	the organization for economic cooperation and development
OCT2	organic cation transporter 2
PMS1	postmeiotic segregation increased 1 (S. cerevisiae)
PMS2	postmeiotic segregation increased 2
p53	a tumor suppressor gene; mass is 43.7 kilo dalton (KDa)
TGF-α	transforming growth factor-α
PDGF	platelet derived growth factor
PlGF	placenta growth factor
pVHL	hippel-lindan tumor suppressor
pKa	negative log of Ka
PK/PD	pharmacokinetic/pharmacodynamic
PKCSM	a computational tool
p300	transcription factor
PGE2	prostaglandin E2
PECAM-1	platelet endothelial cell adhesion molecule
PBS	phosphate buffered saline
pRb-E2F	retinoblastoma protein/ E2F transcription factor
p21	transcription factor 21
QSAR	quantitative structure activity relationship
RMSD	root-mean-square deviation
RT	room temperature
R28	rat retinal precursor cells
ROI	region of interest

ROS	reactive oxygen species
siRNA	small interfering ribonucleic acid
STAT3	signal transducer and activator of transcription-3
SMILES	a computational language
SMARTS	a computational language
SAR	structure activity relationship
Th17	T helper 17
Tsp-1	thrombospondin-1
tRNA	transfer Ribonucleic Acid
TNF–α	tumor necrosis factor
TEER	transepithelial electrical resistance
TPTZ	2,4,6-tripyridyl-s-triazine
TRAIL-4	TNF- related apoptosis inducing ligand-4
U87 MG	glioblastoma IV epithelial cells
USA FDA	food and drug administration of USA
VEGF	vascular endothelial growth factor
VD	volume of distribution
VEGFR	VEGF receptors
WHO	world health organization
XIAP	x-linked inhibitor of apoptosis
v/v	volume by volume
w/v	weight by volume
w/w	weight by weight

LIST OF SYMBOLS

>	greater than
<	less than
≥	geater or equal than
≤	less or equal than
%	percentage
ºC	degree celcius

PEMBANGUNAN DERIVATIF- DERIVATIF ASID ROSMARINIC UNTUK MENSASARKAN IL17A BAGI GLIOBLASTOMA MULTIFORME MELALUI LALUAN ANGIOGENIC

ABSTRAK

Di sini, kami memanfaatkan perencatan glioblastoma melalui kesan IL17A, melalui penyekatan angiogenesis, menggunakan derivatif baru asid rosmarinic (RA). Aktiviti anti-GBM sebatian ditentukan melalui penghijrahan sel, pertumbuhan dalam sel-sel U87 MG, DBTRG MG dan EA.hy926. Aktiviti anti-angiogenik telah ditentukan oleh pembentukan tiub, CAM, dan ujian eksplan aorta. Aktiviti sebatian IL17A dan ekspresi VEGF telah ditentukan dengan menggunakan ELISA, dan aktiviti apoptotic dinilai oleh assai Caspase. Laluan kanser telah ditentukan dengan menggunakan assai pelapur gen. Derivatif menunjukkan > 2 kali ganda kebolehtelapan halangan darah-otak, daripada asid rosmarinik. ROS telah terzahir berasingan selepas rawatan dengan garam dan bes rosmarinate dalam sel U87 MG. IC_{50} garam perak ditemui > 1200 μg / ml, yang tidak boleh ditakrifkan sebagai sebatian toksik. Nar dan FLVM menjejas ekspresi gen PRB-E2F dan MAPK / ERK, dengan masing-masing, 1.03 kali ganda dan 1.14 kali ganda, penurunan. IL17A, VEGF dan protin-protin HIF1α telah dihalang dengan ketara; 2 kali ganda untuk NaR dan AgR, dan 1.5 kali ganda untuk FLVM ($P < 0.0001$). Kami mendapati bahawa pengdeaktifan gen dan protein ini, menyediakan keberkesanan terapeutik melalui aktiviti anti-angiogenik yang diperolehi; 44.01 ± 4.1%, 63.80 ± 4.3%, 61.65 ± 3.9%, dan 46.45 ± 2.8% daripada ciri-ciri anti-migrasi dalam sel-sel U87 MG ($P < 0.05$); 59.83 ± 1.85% , 60.56 ± 4.2%, 79.56 ± 3.65%, 97.34 ± 4.5% daripada ciri-ciri antimigrasi dalam sel-sel EA.hy926 ($P < 0.05$); 69 ± 2%, 95 ± 4%, 81 ± 6.8%, 82 ± 7.8% daripada perencatan pembentukan tiub ($P < 0.05$), dan 86.59 ± 3.45%, 49.69 ±

2.84%, 89.92 ± 4.56%, 58.22 ± 6.47% daripada kesan pencegahan eksplan saluran darah ($P < 0.05$), masing-masing untuk Nar, AGR, FLVM dan FLVZ pada dos-dos yang tinggi. Kami memerhatikan penurunan ketara penanda-penanda-bio apoptotik Bad, Bcl-2 dan Bcl-w dengan 6.53, 1.28 dan 3.97 kali ganda. Pengurangan saiz tumor glioblastoma dan pengambilan makanan haiwan telah diperhatikan, selepas rawatan dengan derivatif-derivatif RA. Keberkesanan anti-glioblastoma ($\Delta T / \Delta C\%$) NaR, AgR, FLVM dan FLVZ, telah didapati masing-masing sebagai 24%, 47%, 2% dan 11%. Kesimpulannya, keputusan- keputusan kajian ini menunjukkan bahawa RA dan derivatif-derivatifnya mempunyai aktiviti antiglioblastoma, dengan mensasarkan ekspresi IL17A dan VEGF, dan sebatian-sebatian tersebut juga menyekat beberapa laluan-laluan berbeza, iaitu laluan-laluan apoptotik dan keradangan.

THE DEVELOPMENT OF ROSMARINIC ACID DERIVATIVES TO TARGET IL17A IN GLIOBALSTOMA MULTIFORME VIA ANGIOGENIC PATHWAYS

ABSTRACT

In this study, glioblastoma inhibition was acheived via the disruption of IL17A, which blocks angiogenesis using new derivatives of rosmarinic acid (RA). Anti-GBM activity of the compounds were determined through cell migration and cell proliferation assays with U87 MG, DBTRG MG, and EA.hy 926 cells. Antiangiogenic activity was investigated with tube formation, CAM, and aortic explant assays. The compounds' activity in IL17A and VEGF expression was determined using ELISA and their apoptotic activity was assessed by the Caspase assay. The cancer pathway was determined using the reporter gene assay. These derivatives showed >2 fold blood-brain barrier permeability compared to that of rosmarinic acid. ROS were differentially expressed after treatment with salt and base rosmarinates in U87 MG cells. The IC_{50} of silver salts was found > 1200 μg/ml, which defined them as non-toxic compounds. NaR and FLVM affected the expression of pRb-E2F and MAPK/ERK genes with 1.03-fold and 1.14-fold downregulation, respectively. The expression of IL17A, VEGF, and HIF1α proteins was significantly inhibited, 2-fold for NaR and AgR and 1.5-fold for FLVM ($P<0.0001$). It is found that inactivation of these genes and proteins provided therapeutic efficacies of antiangiogenic activity at 44.01±4.1%, 63.80±4.3%, 61.65±3.9%, and 46.45±2.8% of antimigratory properties in U87 MG cells ($P<0.05$), 59.83±1.85%, 60.56±4.2%, 79.56±3.65%, 97.34±4.5% of antimigratory properties in EA.hy926 cells ($P<0.05$), 69±2%, 95±4%, 81±6.8%, 82±7.8% of inhibition of in tube formation ($P<0.05$), and 86.59±3.45%, 49.69±2.84%,

89.92±4.56%, 58.22±6.47% of vessel explant prevention ($P<0.05$) for NaR, AgR, FLVM, and FLVZ at high doses, respectively. The downregulation was observed for Bad, Bcl-2, and Bcl-w apoptotic bio-markers at 6.53-fold, 1.28-fold and 3.97-fold activity. The *in-vivo* glioblastoma study using NaR, AgR, FLVM, and FLVZ did not show any drug related adverse effect . The antiglioblastoma efficacy ($\Delta T/\Delta C\%$) of the NaR, AgR, FLVM, and FLVZ were found to be 24%, 47%, 2%, and 11%, respectively ($P<0.0001$). In conclusion, the result of this studies shows that RA and its derevatives have potent antiglioblastoma activity by targeting the IL17A and VEGF expression, and the compounds also blocked a variety of different namely the apoptotic, angiogenesis and inflammatory pathways .

CHAPTER 1

INTRODUCTION

1.1 Glioblastoma, pathology and therapy

Despite many treatment options, cancer remains a growing problem and has become the second leading cause of death globally. Cancer refers to the excessive, uncontrolled growth of abnormal cells, which can invade and eventually destroy other tissues. Furthermore, it can develop in any organ of the body and is life threatening. Notably, each year, more than 5 million people die of cancer (American Cancer Society). Glioblastoma multiforme (GBM), or "glioblastoma" according to the World Health Organization (WHO) classification, also known as Grade IV Astrocytoma is the most common and most aggressive malignant primary brain tumor in humans involving glial cells, and accounts for 52% of all functional tissue brain tumor cases and 20% of all intracranial tumors. Moreover, Glioblastoma is the primary form of brain tumor with approximately 23,000 newly diagnosed cases each year in the United States (Dolecek et al., 2009), while also having a dismal median survival of only 14-15 months (Stupp et al., 2009). In Malaysia, approximately 38000 people die from cancer each year (Malaysia Cancer Index), whereas the incidence of brain and nervous system tumor amounted to 3.3 per 100,000 people (CR) in 2006, as reported by the Malaysian National Cancer Council (MAKNA). GBM is rare and is classified as two categories, either giant cell glioblastoma or gliosarcoma, with incidences of 2–3 cases per 100,000 in Europe and North America (Louis et al., 2016). The rate of occurrence of GBM varies with gender, race, and geographic region. Children under the age of 15 are more frequently diagnosed with primary brain tumors than those between 15 and 19. The survival rate of GBM is low, but early detection accompanied by lifestyle

changes can extend the life expectancy of these patients. Recent research on GBM has provided better understanding of this disease as well as new and better treatment options such as chemotherapy, radiation and surgery. However, treatment of GBM with standard-of-care radiation and chemotherapy with temozolomide or Bevacizumab is still low, with median survival of 15 months.

1.2 How cancer develops

The human body consists of 30 trillion cells which undergo a regular life cycle, with cell death and renewal. This process is tightly regulated in a precise and orderly fashion. Healthy cells have a controlled division system named mitosis, which ensures the needed cell duplication according to the necessity of the organ and tissue in order to maintain their shape and size (Peterson, 2009). Uncontrolled cell division, however, cannot be prevented by normal biological cell functions when system of detection and action cannot work properly. This improper regulation of cell division induces anti-apoptotic signals on the cells, which then can become cancerous. The birth of cancerous cells happens inside the genetic makeup or genome of cells, at the level of individual genes, each on its own, a biochemical instruction to produce each protein in the human body. In fact, the genome is composed of tens of thousands of these genes each encoded into a coiled molecule named deoxyribonucleic acid (DNA) (Peterson, 2009). Genes consist of specific information to make proteins, the very building blocks of cells and controllers of virtually all biochemical and enzymatic reactions in the cells. However, protein function is lost or altered through gene mutation, which can lead to uncontrolled growth, thus inducing formation of tumor masses and cancer development. In this mechanism, cells communicate with each other via receptors on the cell surface, which transfer signals by releasing pro-divide growth factors. This signal diffuses into the nucleus where genes are located, which

then activates the proto-oncogenes to cause mutation in normal cells. in normal cases, growth signaling goes to nucleus and induces growth pathways in the genome, however when there is mutations in the pathway, the growth signaling can be hyper activated or sustained in absence of outside stimulus. This mutation signaling activity permits the growth and division of the cells repeatedly with or without the growth factors. In addition, tumor suppressor genes, which halt growth, can become mutated due to carcinogenic factors thus stopping their growth inhibitory activity (Peterson, 2009). The cause of tumor formation is not only the mutation of tumor suppressor genes, but also of other safety mechanisms in the cells. Furthermore, abnormal cell cycle function or abnormal timing of the biological clock through different mutations, could, via inactivating and activating proto-oncogenes and tumor suppressor genes respectively, while in a healthy cell could derail the cells normal division leading to cancer formation (Peterson, 2009). In normal cells, the clock mechanism can detect impaired cycling activity and can seek to repair it by triggering the tumor suppressor gene p53 in order to provide pro-apoptotic signals to stop uncontrolled cell division (Peterson, 2009). Unfortunately, in cancer cells for example, p53 might be mutated due to carcinogenic effects, thus this safety mechanism could not function. Lacking these safety mechanisms, cells continue to replicate, which could also lead to improper DNA amounts in cells. Normal cells can undergo roughly 40 cell divisions, after which "telomeres" become too short, which could lead to loss of DNA sequences in subsequent replication cycles which ultimately kills the cells. These telomeres or sequences at the end of DNA to protect them and work to regulate irregular cell division. In cancer cells, the telomerase enzyme responsible for lengthening the telomeres, an enzyme usually only active in the germline, is re-expressed which leads to cells "immortality" thus perpetuating the uncontrolled duplication of cancer cells.

However, the circumstances above (i.e. gene mutation and telomerase reactivation) are not on their own sufficient for the development of cancer, because a combination of other biological obstacles need to be overcome.

Normal cells build up a fibrous meshwork called the extracellular matrix on which to grow and form organs and other complex tissues and cannot survive without this matrix, while cancer cells can live without it. This irregular growth of cancer cells forms tumors which can grow on top of each other, creating a mass of cells. Also, unlike normal cells, tumor cells can develop their own network of blood vessels to supply the blood, a process called angiogenesis (Peterson, 2009).

Tumors may be benign or malignant depending on their tissue invading capabilities. Benign tumors cannot invade normal tissues and are limited to one site but malignant tumors can invade normal tissues and travel to distant locations in the body through the blood vessels. Tumor cells then can pass into the lymphatic system and metastasize. While circulating, tumor cells home towards tissues expressing compatible receptors, thus leading to tumor metastasis in specific tissues. During this metastatic phase of cancer, many cells die in the bloodstream or become dormant and again start to grow, for reasons not yet known (Peterson, 2009).

1.3 Causes of cancer

The causes of cancer are not yet fully understood, but general causes include life style, chemical exposure, environmental, pathological factors and in addition the inclination of some people to develop the disease more than others. Prolonged exposure to carcinogens from chemical, biological and physical industry causes the

cellular damage in personnel and this exposure might increase the production of free radicals in the body which are known to damage DNA by taking negatively charged particles i.e. electrons which cause mutations. Smoking is another cause of cancer related to lung, oesophagus, respiratory tract, bladder, pancreas, and that might be cancers of the stomach, liver, and kidneys (Peterson, 2009). In addition, obesity can increase the likelihood of developing cancer and some microorganisms such as human papillomavirus (HPV), Hepatitis B and C viruses, Epstein-Barr virus, Human Immunodeficiency Virus (HIV), *Helicobacter pylori*, and polyomavirus are linked to cancers in the cervix, liver, lymphatic system, circulatory system, stomach and skin cancer, respectively (Peterson, 2009). People in third world countries are suffering from liver cancer and bladder cancer due to infections of parasitic organism such as *Clonorchis sinensis* and *Schistosoma haematobium.* Environmental factors such as sunlight, X rays, and ultraviolet (UV) radiation, working in radioactive mines and radon gases have been linked with skin cancer (Peterson, 2009). Additionally, pollutants in the air, soil and water are linked with bladder cancer. In the chemical industry, personnel are exposed to carcinogenic chemicals such as benzene, asbestos, vinyl chloride, aniline dyes, arsenic, and certain petroleum products (Peterson, 2009). Consistently, cancer is developed due to some genetic mutations in tumor suppressor genes of BRCA1 or BRCA2 for breast cancer at the age of 70 and the mutations of MSH2, MLH1, PMS1, and PMS2 genes are major players in colon cancer (Peterson, 2009). Breast and uterine cancer are also developed due to long exposure of estrogen hormones. Other reasons for breast cancer are early menstruation, late menopause, no children, or having children after the age of 30. In addition, male testosterone also plays a role in the developing of cancer in the male reproductive organs. Statistics show that black women have a lower risk of developing breast cancer compared to

white women. Furthermore, Hispanic, Asian and North American women have the lowest risk for breast cancer worldwide, while African American men are more likely to develop cancer, thus demonstrating the impact of genetic makeups on the propensity for developing cancer.

1.4 Types of cancer

More than 100 types of cancers are found in various organs of the body. The nature of cancer is defined based on the origin of the cancer, how it first formed and the types of cells involved in the tumor. In addition, metastasis in a tissue are not defined as new cancers but rather metastatic cancers of the original tumor type. For example, breast cancer describes any cancer that originated in the breast. If the cancer spreads to a new organ, such as the lungs, the lung tumor is named metastatic breast cancer, rather than lung cancer. Most cancer patients suffer from cancers of the prostate, breast, lung, colon, lymphoma, bladder, uterine, skin, kidney, leukemia, pancreas, ovaries and stomach.

1.5 Treatment

Treatment of cancer is dependent on the type and stage of the tumor development. Several treatments have been created to treat cancer however no magic pill cure for cancer exists. These treatments include surgery, radiation, chemotherapy and immunotherapy, which have been shown to extend the life expectancy of patients. Early detection of any tumor may provide a higher scope in eradicating the chance of cancer development. The detailed recent therapeutic information can be found at Academy of Medicine of Malaysia - Clinical Practice Guidelines (CPGs).

1.6 Angiogenesis and cancer

Tumors develop their own network of blood vessels, a process called angiogenesis. Angiogenesis is a normal process in the body which is classified as pro- and anti-angiogenic factors. There is a balance in normal tissue between pro- and anti-angiogenic growth factors which is defined as an angiogenic switch (Hananah and Folkman, 1996). This natural balance can stimulate vascular growth when necessary. The principle angiogenic factor is vascular endothelial growth factor (VEGF). Other factors involved in this process are transforming growth factor-α (TGF-α), hepatocyte growth factor (HGF), Basic fibroblast growth factor (bFGF), angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), platelet derived growth factor (PDGF), matrix metalloproteinases (MMP), and placenta growth factor (PlGF) etc (Peterson Karen, 2009). The angiogenic switch is controlled by inhibition of these pro-angiogenic factors by some endogenous anti-angiogenic factors, namely endostatin, angiostatin, and thrombospondin-1 (Tsp-1), by promoting apoptosis. Further, angiogenic growth factors are influenced by the hypoxia in the tumor microenvironment. This environmental response is activated by the Hypoxia induced growth factor 1α (HIF-1α) (Naumov et al., 2008; NIH, 2009). Normally, HIF-1α is ubiquitinated in the presence of von Hippel-Lindan tumor suppressor (pVHL) protein. This HIF-1α protein is then degraded, thus stopping its signaling, but this degradation is stopped under hypoxic condition where HIF-1α interacts with the p300 and cAMP responsive element binding protein (CREB) proteins. The HIF-1α complex enters into the nucleus and heterodimerizes with HIF-β, which transcripts the genes of interest (Courtwright et al., 2009; Kang et al., 2009; Ribatti et al., 2007; Wouters et al., 2008; Land and Tee, 2007). HIF-1α can induce the expression of growth factors of VEGF, PDGF, and TGF-α by binding with the hypoxia responsive element the promoters of

these growth factor genes and inducing their expression. Other factors such as mammalian target of rapamycin (mTOR) can also interact with HIF-1α (Vadysirisack and Ellisen, 2012). This signaling molecule forms a complex which is phosphorylated and affects cell growth. Hypoxia also regulates mRNA translation and promotes tumor formation. Pro-apoptotic factors such as BH-interacting domain death agonist (BID), Bcl-2 antagonist of cell death (BAD), and Bcl-2 associated X protein (BAX) are inhibited in hypoxic conditions. This increases the cell-life span and tumor growth. VEGF signals cells to form capillary-like blood vessel structures with the aid of endothelial cells and to damage the extracellular matrix with some enzymes. VEGF is expressed and helps to induce the anti-apoptotic proteins Bcl-2 and its homolog A1-10 VEGF receptors (VEGFR). VEGFR phosphorylates tyrosine residues on several targets to activate downstream signal transduction molecules. Tumor microenvironment stimulates angiogenic factors to attract the endothelial cells to grow the blood vessels surrounding the tumor. The mechanism of vessel growth is discussed below.

Embryonic development of neovascularization of tumor angiogenesis is defined as vusculogenesis that is developed through endothelial cell production from angioblast. Characteristically, vusculogenesis induces varieties of angiogenic signals from preexisting blood vessels. Endothelial cells produce proangiogenic factors to form the provisional tubes and novice perivascular cells which provide them maturity and stability. Usually, tumor angiogenesis induces a progression of new vessels from preexisting vessels and the tumor recruits signal from the bone marrow and tumor stem cells (Herbert and Stainier, 2011) to induce the formation of more blood cells. The tumor functional network effects the disease development and creates short term

effects via different signaling molecules and vascular renovation, wherein suppression of one pathway can stimulate others. Termination of this vascular network has been a therapeutic strategy as an anticancer target. Controlling the signaling pathways of neovascularization in downstream signaling molecules could be the new strategy to suppress angiogenesis. The continuous progression of the tumor lesion is influenced by hypoxia and nutrient deprivation, which can generate more angiogenesis for further survival of tumor cells. Different kinds of cytokines and growth factors are released from the tumor microenvironment to activate different cascades of angiogenic events. For example, VEGF is released from the tumor which stimulates endothelial cell proliferation. On the other hand, PDGF stimulates perivascular cells and modulates vascular stability and growth of tumor (Weis and Cheresh, 2005). Moreover, development of the tumor vascular bed is driven through tumor associated fibroblasts to provide the extracellular matrix (ECM) proteins. Tumstatin and endostatin act as endogeneous inhibitors against angiogenesis through inhibition of ECM proteins. Targeting of angiogenesis signaling proteins needs an anti-angiogenic efficient drug with complementary effect of systematic apoptosis (Franco et al., 2010; Demaria et al., 2010; Grivennikov et al., 2010).

In angiogenic processes different independent and interdependent factors are observed such as Notch, semaphorins, ephrine and slits which are GF family proteins comprised of SLIT1, SLIT2, and SLIT3 ligands. These ligands are recognized by ROBO1, ROBO2, ROBO3 and ROBO4 receptors (Jones et al., 2008). Tumor cell expresses these ligands and their receptors as paracrine and autocrine factors. Guidance molecules are activated through their receptors that recruit progenitor cells, which induce the tumorigenic process through macrophage signaling pathway.

Potential anti-angiogenic ligands can bind to these receptors to inhibit other angiogenic signaling pathways. For example, ROBO4 inhibits VEGF and FGF pathways and thus the mechanism of guidance molecules in angiogenic activity and their role with other angiogenic growth factors might be a potential therapeutic strategy (Park et al., 2003; Zygmunt et al., 2011). Furthermore, integrins are another type of ECM protein which can control the angioganic cascade. Integrins are primary cell matrix protein with which other ECM intracellular signaling pathways are controlled. It has α and β subunits which can bind with multiple ECM proteins. Ligation of integrin mediated signaling could crosstalk between integrin and other activated cytokines (Kim et al., 2011). Conversely, the overexpression of integrins can promote the proteolytic activation of ECM protein which increases the endogenous angiogenesis through matrix formation. Recent studies have shown that cyclic peptide could trigger $\beta_v\alpha_3$ type integrin to block angiogenesis. Expression of $\beta_v\alpha_3$ can increase tumor growth and metastasis. It was also found that high grade glioblastoma brain tumor overexpress $\beta_v\alpha_3$ receptor and ECM vitronectin. Also, cyclic peptide antagonist shows potential inhibitory activity against orthotopic glioblastomas in mice model. Cilengitide is the first integrin antagonist approved by the US food and drug administration (FDA) for cancer therapy currently in under clinical trial. Additionally, proangigenic integrins are currently considered for clinically effective anticancer drugs which might target α_v, $\alpha_v\beta_3$, $\alpha_5\beta_1$, $\alpha_v\beta_3$ $\alpha_v\beta_5$ and $\alpha_5\beta_1$ coupled with Arg-Gly-Asp (Desgrosellier and Cheresh, 2010). Cross talk between integrin and GF receptors are widely studied recently. VEGF, FGF, PDGF and angioprotein are proangiogenic proteins and each of them has specific isoform such as VEGFA-121, VEGFA-165, VEGFA-189 and VEGFA-206. The activity of VEGF receptors such as VEGFR1, VEGFR2 are modulated by clustering or dimerization of these receptors.

Integrin and GF signaling proteins show an interaction between $\alpha_v\beta_3$ with VEGFR2, HGFR c-Met, FGFR1, PDGFR, EGFR and IGF-1R. The prevention of this interaction could reduce the angiogenesis in tumor (Meyer et al., 2011).

Extra cellular matrix proteins are crucial for controlling angiogenesis because MMPs such as MMP-2, MMP-9, MT1-MMP trigger the angiogenesis process via the destruction of ECM proteins. MMP mediated degradation can affect cryptic Arg-Gly-Asp sites of integrin proteins against sprouting of endothelium that selectively can damage ECM remodeling. Understanding the mechanism of the MMP enzymes could be another therapeutic strategy to target tumor angiogenesis (Deryugina and Quigley et al., 2010; Sounni et al., 2011).

MicroRNAs is another kind of small noncoding RNA which can act as an angiogenic switch for vascular developments through protein translation regulation. Different kinds of RNA binding proteins regulate miRNA function by controlling biogenesis. Targeting of miRNA could not be selective therapeutic approach because of its direct relationship with tumor suppressor genes. VEGF expression competitively depends on miRNA function (Huynh et al., 2011). Recently, some angiogenesis-controlling miRNA called angiomiRs were identified. MiR-126, miR-130a, miR-210 and miR-296 stimulate angiogenesis whereas miR-221 and miR-222 suppress tumor angiogenesis. These angiomiRs regulate migration, proliferation, and hypoxia of tumor cells and targeting of angiomiRs could be a potential target to control cancer progression. The angiomiR mir-126 controls angiogenesis by upregulation of miRNAs, which regulates the angiogenic switch by suppressing tumor promoting genes. miRNA inhibitors can decrease the miRNA expression and control angiogenic

response. Such is the case for example pertaining to the involvement of miR-132 in inducing the endothelial proliferation through p120RasGAP expression. Anti-miR-132 therapy could inhibit endothelial cell proliferation via affecting p120RasGAP expression and suppression of Ras activity. In breast cancer cell, miR-20b regulates VEGF expression by triggering HIF-1α. miR-93 reduces expression of $\alpha_v\beta_8$ integrin in glioblastoma cell which results in the growth of endothelial cells to promote angiogenesis. Blocking of angiogenesis using anti-miRNA therapy requires delivery of new RNA into the tumor as the therapeutic approach. However, recent study showing the use of such therapy through adenovirus-mediated miRNA delivery have revealed that the therapy might have some potential efficacy in preclinical models but its application in humans might be complex because miRNA or anti-miRNA delivery needs further validation in better disease models. In addition, preparation of nanoparticles containing the anti-miRNA molecules could be an interesting delivery mechanism into the tumor cells, but overall, further development is needed to address the use of miRNA therapy (Murphy et al., 2008).

Anti-angiogenic therapy does not only block signaling pathways but also provides inverse effects to other cells. Co-culturing of endothelial cell and smooth muscle cell shows potential inhibition of cell proliferation but this study shows that after treating the cells, endothelial cell pericyte could be reformed for further survival of cell and thus this strategy might not suitable for cancer cell. In addition, inverse effects of anticancer therapy are a particular problem in integrin and miRNA-associated tumorigenesis. Although a similar dichotomy exists in VEGF associated tumorigenesis, VEGF-inhibited VSMC activity shows potential PDGF inhibition of angiogenesic processes, and that VEGF activated VEGFR2 could induce PDGFRβ

signaling through interaction of VEFR2-PDGFRβ complex. This mechanism postulates that the use of anti-angiogenic therapy might provide complementary efficacy by inducing cancer cell apoptosis by inhibiting the proangiogenic cytokines (Hu et al., 2009). Further, antibody therapy have shown efficacy in the inhibition of endothelial cell migration and tumor infiltrating growth factors in esophageal cancer. Despite the usefulness of antibody therapy, further investigation is warranted to authenticate its clinical efficacy in tumor microenvironment (Chung et al., 2010, Lu et al., 2000). The discovery of angiogenesis pathways has helped to develop many FDA-approved anticancer and anti-angiogenic drugs, such as avastin, sorafenib, and sunitinib that target VEGF-A, Raf and PDGF, respectively. It is notable that combination therapy could be good strategy to block angiogenic signaling pathways and to prevent high levels of mutation and survival factors in the tumor. To improve the anticancer drug efficiency, anti-VEGF drugs are administered with chemotherapeutic agents to provide high clinical potency. The perivascular compartment could also be beneficial for treating angiogenesis due to its activity in vessel maturation and blood flow (Hu et al., 2009; Chung et al., 2010; Azzi et al., 2013).

The tumor blood vessels can stimulate the tumor microenvironment and govern the mutated signaling pathways. Tumor-associated endothelial cells show more differential properties than normal endothelial cells which is a useful tool to detect the cancer for targeted therapies, and many methods are being developed to identify the cancer using the tumor associated vasculogenesis properties. The results of flow cytometry and magnetic bead separation show that tumor cells attract the

endothelial cells to increase angiogenesis, survival activity, immortality and chemoresistance activity.

Many studies show that angiogenesis and immunosuppression instantaneously occur instantaneously during aseptic tissue injury resulting from ischemia, reperfusion injury, infection and pregnancy. This dual biological changes is found in tumor microenvironment and is initiated by the induction of complex cellular processes. Myeloid cells promote immunosuppression and angiogenesis in tumors and myeloid derived suppressor cell (MDSC) are noticeably increased in tumor patients (Motz GT, and Coukos G., 2011). This has been shown to be mediated through T and NK cell activation and results the high level of NO, ROS, interlukin-10, transforming growth factor-β (TGFβ). It has been demonstrated that MDSC can directly promote angiogenesis and thus a study designed to target the MDSC shows that treatment with BV8 antibody deceased MDSC in the tumor-bearing mice. Some myeloid cell subsets promote angiogenesis, plasmacytoid DC, tumor associated macrophages (TAM) and monocytes mediated angiopoetin (Bourbie-Vaudaine et al., 2006) which have the ability to promote expression of VEGF, FGF, CXC-chemokine ligand 8 and COX2, thereby upregulating the immune-stimulatory factors such as lymphocytes cell secreted cytokines. It has been suggested that $CD^{+}CD25^{+}FOXP_3^{+}T_{Reg}$ cells can make home to tumor sites and play a role in hypoxia induced angiogenesis in ovarian cancer progression. The T_{Reg} complex (receptor complex) can upregulate the expression of CC-chemokine ligand 28 (CCL28) to recruit $CD4^{+}CD25^{+}FOXP_3^{+}T_{Reg}$ cells from peripheral blood flow. Overexpression of CCL28 can recruit T_{Reg} through increasing angiogenesis and immunosuppressive microenvironment via VEGFA. VEGFA induces hypoxia through $CD4^{+}CD25^{+}T_{Reg}$ and promotes cell proliferation. In addition,

$CD4^+$T cells induce the expression of neuropilin 1 (NRP1) in dendritic cells (DC) through trogocytosis (Facciabene et al., 2011; Sarris et al., 2008). DC then secrete VEGFA in tumor sites and increases angiogenesis. Other immune cell subsets include B cell, Natural killer T (NKT) cells, NK cells, γδ T cells have been shown to produce VEGFA. Mesenchymal stem cells (MSC) are a type of stromal cells that can secrete VEGFA by differentiating cancer associated fibroblast (CAF). MSCs promote angiogenesis by secreting VEGFA, thus inducing the differentiation of CAFs which express α-smooth muscle actin, and TIE2. Total contribution of MSCs is unknown but it has an integral role in the establishment of the tumor microenvironment, supporting both immunosuppression and angiogenesis. Stromal CAF cell are activated by TGFβ, FGF, and PDGF and can secrete VEGFA to promote the recruitment of cells of the myeloid lineage through CCL2 and CXCL12 activation. VEGFA can control a varied assortment of immune functions and serves as a prototypical molecules to mediate angiogenesis. The overexpression of VEGFA by tumor cells can produce high intratumoural T_{Reg} cells in order to provide tolerogenic situation and tumor evasion (Bhowmick et al., 2004).

Endothelial cells (ECs) can regulate immune cells by controlling leukocyte trafficking by inducing leukocyte extravasation to the tumor site. This activity of ECs is mediated through intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1). ICAM1 and VCAM1 can moderate the adhesion and migration of leukocytes. VEGFA and bFGF can increase the adhesion of T cells through tumor necrosis factor (TNF). TNF can increase the VCAM1 and ICAM1 expression via Caveolin 1. T cells are involved in the activation of endothelium B receptor (ETBR) which is upregulated in ovarian cancer and causes inflammation and

a quiescent tumor endothelium phenotype. Tumor endothelial cells attract immune cells through tumor reactive T_{Eff} cells and NK cells. Furthermore, T_{Reg} cells migrate to the tumor via VEGFA and hepatocyte growth factor (HGF) signaling in hepatic carcinoma cells through ubiquitously expressed endothelial and vascular endothelial receptor 1. There are several mediators (signaling molecule) of endothelium including PDL1, PDL2, FAS ligand, TNF-related apoptosis inducing ligand (TRAIL) and endothelium cell marker CD31 which are responsible for extravasation of leukocytes. IL-6, IL-10, TGFβ and PGE2 are several of the endothelial cell mediators that can suppress immune response (Dirkx et al., 2003). T cell immunoglobulin domain and mucin domain protein 3 (MD3) are expressed in endothelium through the activation of signal transducer and activator of transcription 3 (STAT3). Endothelial cell can express ICOS ligand (ICOSL), CD40, CD58, CD80, CD86, CD137, MHC class I and class II molecules to upregulate angiostatic, and TH1 cell-associated cytokines. The angiogenic mechanism of these molecules is still not clear but many studies show their involvement in angiogenesis. Also, the mural cells, a type of vascular cells are involved in angiogenesis and immunosuppression for blood vessel formation and healing. It is postulated that immunosuppression and angiogenesis require different growth factors and inflammatory cytokines depending on the expression of cellular signals to promote tumorigenesis. In hypoxic state of tumor, IL-6 synergizes with VEGFA expression through VTCN1 and promote immunosuppression, and T_H17 differentiation (Choi et al., 2004; Pucci et al., 2009).

The tumor microenvironment can stimulate homeostatic tissue repair activity that can be reflected as either angiogenesis or immunosuppressive. The use of anti-angiogenic therapy in combination with chemotherapeutic agents can potently affect

tumor growth and can reduce the side effects of the toxic drugs. Moreover, lysate vaccine and ETBR therapy is currently being tested in preclinical model as a potent anti-angiogenic therapy. This vaccine is used with COX2 inhibitor and anti-VEGF antibody. Oxidized lipids which act as endogenous ligands for the TLR2-MYD88 pathway, controls wound healing, ischemia and tumor angiogenesis by stimulating endothelium cells (Kiichiro Yano et al., 2006; Cavassani et al., 2010).

1.7 Interleukin 17A, angiogenesis, inflammation and cancer

Interleukin 17 comprises six members (IL17A – F) with the most heavily studied being IL17A. IL17A is produced by Th17 cells (Dong et al., 2008) and shows potential roles in promoting tumor angiogenesis and inflammation (**Figure 1.1**). In tumor pathogenesis, chronic inflammation has a significant role in inducing tumor growth (Coussens et al., 2002), wherein IL17A acts as proinflammatory factor in cancer patients (Fujino et al., 2003). In the tumor, IL17A induces blood vessel formation and angiogenesis (Lemancewicz et al., 2012; Liu et al., 2011) and causes T-cell dependent tumor development and the Th17-produced IL17A increases the pathogenesis of the cancer (Dong et al., 2008).

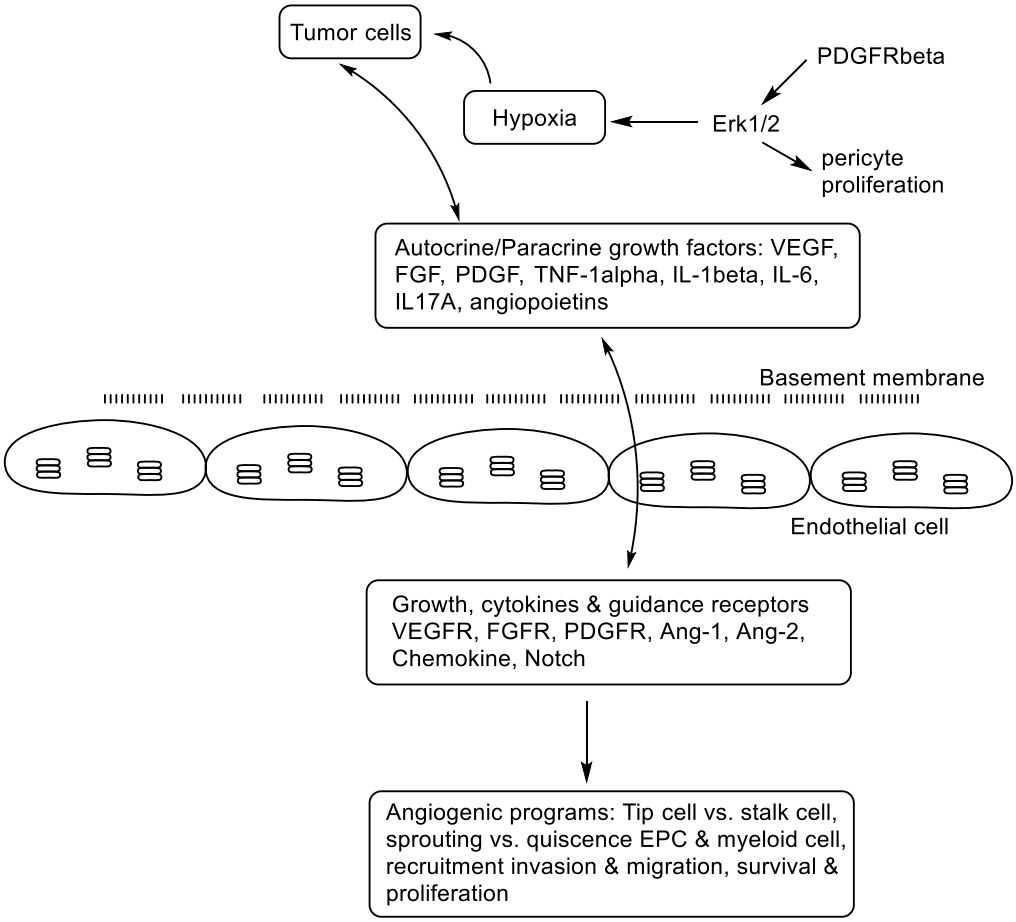

Figure 1.1 Effect of tumor cells in angiogenesis in the tumor microenvironment. Multiple factors are involved in the tumor-induced neovascularization. These factors (autocrine and paracrine growth factors) transform the normal epithelial cells into tumor cells. During this process, hypoxia inducible factor HIF1α and HIF1β trigger angiogenesis processes. As a result, tumor cells release soluble factors, cytokines and inflammatory cytokines (IL17A, IL6, etc.) to induce the sprouting, proliferation and migration of quiescent endothelial cells near the blood vessels and lymphatics.

IL17A is also, termed as cytotoxic T-lymphocyte–associated antigen 8 (CTLA–8) (Rouvier et al., 1993) which is mainly produced by the activated CD4 T cells (Yao et al., 1995) and induces the expression of IL-6, IL-8, prostaglandin E2 (PGE2), and intercellular adhesion molecule 1 (ICAM–1) (Yao et al., 1995; Fossiez et al., 1996; Aarvak et al., 1999). Moreover, IL17A induces a higher expression of tumor necrosis factor α (TNF–α), IL-1 and stromelysin by macrophages (Jovanovic et al., 1998). This in turn, activates IL-17R (type 1 transmembrane protein) leading to activation of the transcription factor nuclear factor B, and regulation of extracellular-regulated kinase 1 (ERK1), ERK2, c-Jun N-terminal kinase (JNK), and p38 mitogen–activated protein kinases (Yao et al., 1995; Shalom-Barak et al., 1998; Awane et al., 1999). There are some homology between IL17A members but, IL17AR has no homology making it a novel receptor family. Although the expression of IL17A is found in normal and cancer cells, the particular activity in cancer is not clear yet. CD4 T cell derived IL17A is stimulator of angiogenesis and has a potential role in neovascularization via migration of endothelial cell and cord formation. IL17A might have an indirect or direct proangiogenic effects in the tumor microenvironment by inducing TGF-β and PDGFb (Brogi et al., 1994). IL17A also has role in fibroblast-induced neovessel formation in inflammation and tumors and fibroblasts have been shown to modulate hypoxia and thereby tumor inflammation (Volpert et al., 1997; Cho et al., 2000). However, IL17A provides pro-angiogenic activity in tumors according to the immunogenicity and types of tumor cells. The development of tumors through angiogenesis is mainly regulated because of alteration of these pro- and anti-angiogenic factors by the influence of IL17A which causes an imbalance of angiogenesis factors and growth factors in the vascular microenvironment. This

activity of IL17A could be controlled by targeting the CD4 T cell derived IL17A in the tumor microenvironment.

1.8 Interleukin 17A and glioblastoma multiforme: pathology and treatment

Glioblastoma Multiforme (GBM) is the most frequently occurring primary brain tumor. GBM progresses very quickly and patient's median survival rate is 12 – 15 months. Inflammation and angiogenesis have potential impacts on the formation of brain tumors (Guiton et al., 2010; Smith et al., 2010). In glioblastoma, IL17A signaling or elevated IL17A levels are observed due to the dysfunction of T helper cells (Andaloussi et al., 2008; Prahlad et al., 2013; Julian et al., 2013; Jinhui et al., 2013) and infiltration of immunosuppressive microglia and macrophage cells (Watters et al., 2005). IL17A also activates platelet endothelial cell adhesion molecule (PECAM-1), also known as cluster of differentiation 31 (CD31) cells as well as the IL-6–STAT3 signaling pathway. It's also been shown to suppress cytotoxic T lymphocytes by reducing their cytotoxic effect via the support of CD8 (Nam et al., 2008; Toh et al., 2009; Wang et al., 2009; Stewart et al., 2006). The secretion of IL17A from astrocytes (Tzartos et al., 2008; Li et al., 2005) is augmented by the recruitment of Th17 polarized $CD4^+$ T-cells (Brucklacher-Waldert et al., 2009; Hofstetter et al., 2009), $CD8^+$ T-cells, gamma delta T-cells (Sutton et al. 2009), NK-cells (Rachitskaya et al., 2008), and granulocytes (Li et al., 2010; Hoshino et al., 2008) to the central nervous system (CNS). This is further modulated by IL-23 signaling (Langrish et al., 2010; Park et al., 2005; Harrington et al., 2005). In addition, TGF-b, IL-6, and IL-21 have been shown to be the main causes of high expression of IL17A, this being produced due to the synergistic activity of Th17 cell in association with the pro-inflammatory cytokines (Veldhoen et al., 2006; Bettelli et al., 2006; Mangan et al.,

2006; Korn et al., 2007). IL17AR, which is expressed in the CNS on astrocytes, microglia and endothelial cells (Sarma et al., 2009; Kebir et al., 2007) activates NFkappaB and MAPK via TRAF6. The adaptor protein Act-1 is also activated (Chang et al., 2006; Qian et al., 2007), thereby further exascerbating inflammation by the involvement of proinflammatory cytokines, chemokines and antimicrobial peptides such as G-CSF and the ELR^+ members of the CXC family of chemokines CXCL1 and CXCL2 (Ouyang et al., 2008; Carlson et al., 2008; Fossiez et al., 1996; Kanget et al., 2010) through the recruitment of neutrophils. Astrocytes, a subtype of glial cells demonstrate high expression of IL17A receptor through a synergistic interaction between IL-6 and IL17A, which activates the microglia in astrocytes (Kawanokuchi et al., 2008; Ma et al., 2010), mediated by the Act-1 (Kanget et al., 2010). In the inflammatory and tumor microenvironment, heat-shock protein 90 (Hsp90) and Act1-interacting protein induce high levels of IL17A. IL-17 induced signaling and gene expression are abolished due to IL17A dependent phosphorylation. This phosphorylation is due to inhibition of Hsp90 chaperone function by the loss of interaction between Hsp90 and IL17A (Toh et al. 2009). IL17A promotes the upregulation of pro-inflammatory and neutrophil-mobilizing cytokines and chemokines including JAK2/STAT3, MAPK, NF-κB, IL-6, IL-8, MMP2, VEGF, GM-CSF, GCSF, TNF-α, TGF-β and IL-1β. In addition, Cdc37, together with the chaperon Hsp90 can protect Akt from proteasome-mediated degradation (Sato et al., 2000 and Basso et al., 2002) by forming the Akt-Hsp90 complex. A study by Wang et al. 2002 found both Hsp90 and Cdc37 in the IKK complex (Wang et al. 2012). Further, p23 acts as co-chaperone in the Hsp90 chaperon system, but is specific for steroid receptors and fibrillization of the protein. The above discussion postulates that the molecular targets for the inhibition of the IKK complex or inactivation of Cdc37

and p23, both co-chaperones of HSP90, might suppress the IL-17, IL-6 and VEGF activity.

The inflammatory cytokines can invariably infiltrate the blood-brain barrier (BBB) and cause severe reactions by increasing inflammation and pain due to glioma growth. IL17A might penetrate the BBB by releasing reactive oxygen species (ROS) further enhancing angiogenesis and thereby blood supply to tumor (Chang et al., 2006; Huppert et al., 2010). Proinflammatory cytokines and chemokines are upregulated by the stimulation of IL17A (Cua et al., 2003; Kebiret al., 2007), thus mediating chronic and acute vascular inflammation. The relationship between the CNS vascular pathology and IL17A is mediated by malformations of arteriovenous and polymorphism of IL17A gene (Jiang et al., 2011). The vascular pathology is mediated by the TGF-β signaling as upstream regulator of IL17A (Bettelli et al., 2006; Mangan et al., 2006; Korn et al., 2007; Sarma et al., 2009). In addition, high expression of ROS in the brain endothelial cells downregulates occludin which is a vital signaling molecule in cellular tight junctions of blood vessels and brain cells, further increasing the permeability of IL17A to the brain and exacerbating the cycle in the tumor. (Huppert et al., 2010). Aside from the above molecular interaction of IL17A, it was shown that EGFRvIII is one of highly expressed potential molecules in GBM tumorigenesis and could be a potential target by inhibiting the IL17A, IL-6 and IL-8 activity (De Fazio et al., 2012).

1.9 Natural compound as anti-angiogenic drug

Plant chemicals including secondary metabolites has been widely investigated for the treatment of angiogenesis based diseases. The most useful and studied natural

products are phenolic or polyphenolic compounds which show anti-angiogenic properties with potential anticancer efficacy. The mechanism of action of natural products was previously unknown which lead to their poor acceptance in main regulatory bodies. Recent observations however, and wide array of studies have shown their specific interference with biological targets at the molecular level, thus leading to a significantly increased rate of approval and use worldwide. In particular, natural phenolic chemicals such as flavonoids, rosmarinic acid derivatives, caffeic acid derivatives and diarylheptanoids are found to have potential anti-inflammatory activity, chemoprevention and cytoprotection activity have a pleiotropic influence on cellular signaling towards VEGF, NF-κB or Nrf_2 and oxidative effects of cancer (Prasad et al., 2010; Rahman et al., 2006; Pietta, 2000) (Table 1.1), all with minimal side-effects. Moreover, the natural chemicals found in many fruits and vegetable provide long-term health and nutrition due to their ample amounts in everyday foods. These phenolic chemicals have substantial benefits to health which reinforces a balanced angiogenesis in the human body. There are many excellent anti-angiogenic natural compounds found in vegetables containing a phenolic substructure. Among these compounds, six flavonoids from different subclasses: quercetin, fisetin, epigallocatechin-3-*O*-gallate, xanthohumol, (2*S*)-7,2',4'-trihydroxy-5-methoxy-8-dimethylallylflavanone and genistein have been highlighted here to discuss their potential anticancer effect through disruption of angiogenesis pharmacology (Table 1.2). The angiogenic properties of the natural chemicals should be specific and non-toxic, and potent enough to realistically speculate on the anti-angiogenic activity in vivo. The endothelial cells (ECs) are directly involved in the angiogenesis process due to a plethora of signaling cytokines from cancer tumors. These cells have thus been

given special attention for testing therapeutic efficacy of these anti-angiogenic compounds to verify for unspecific cytotoxic effects.

Table 1.1 Natural phenolic compounds with anti-angiogenic activity and their evaluated molecular mechanisms of anti-angiogenesis (Qiu et al., 2015).

Compound name	Mechanisms of anti-angiogenic action
4-Hydroxybenzyl alcohol	Downregulation of VEGF and MMP9 protein expression
Curcumin	Reduction of VEGF expression, inhibition of transcription factors, mTOR pathway and MMP9 protein expression
Ellagic acid	Inhibition of VEGF and PDGF receptor phosphorylation
Resveratrol	Abrogation of VEGF-mediated tyrosine phosphorylation of vascular endothelial (VE)-cadherin, inhibition of VEGF-induced and FGF-2 neovascularization
Quinoline-substituted phenols	Inhibition of VEGF and Transforming Growth Factor-β1 (TGF-β1) expression
4-Amino-2-sulfanylphenol derivatives	Inhibition of protein kinase B/Akt and ABL tyrosine kinase
Natural-like acylphloroglucinol derivatives	Under investigation
Epigallocatechin gallate (EGCG)	Inhibition of estrogen-stimulated VEGF expression, HIF-1α and NF-κB, inhibition of MMP-2 and MMP-9, inhibition of urokinase plasminogen activator.
Xanthohumol	Inhibition of NF-κB and Akt pathways
Genistein	Inhibition of VEGF and HIF-1α protein expression
Fisetin	Downregulation of VEGF and eNOS expression, inhibition of MMPs function
Quercetin	Inhibition of the expression of VEGF-2, inhibition of COX-2 and arachidonate 5-lipoxygenase (LOX-5), inhibition of NF-κB, In some cell types it activates angiogenesis.
(2S)-7,2',4'-Trihydroxy-5-methoxy-8-(dimethylallyl)flavanone	Downregulation of reactive oxygen species (ROS) levels and VEGF expression